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Human Probiotic Potentials of Lactobacillus Tucceti CECT 5920 and Lactobacillus Mindensis TMW Isolated from Nigerian Fermented Foods

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Abstract

In vitro Probiotic potentials of Lactic acid bacteria (LAB) isolated from traditional fermented foods namely: ugba, ogi, fermenting cassava and kunu-zaki were studied. 25 samples of each of the four types of fermented foods were serially diluted in sterile peptone water 0.1ml aliquots of appropriate dilution was streaked on De Man Rogosa Sharpe (MRS) agar containing 50mg of nystatin for the isolation of LAB. 48 LAB were isolated from the samples these were screened for bacteriocin production by the Agar Well Diffusion assay and two best bacteriocin producers characterized by molecular method as Lactobacillus tucceti CECT 5920 and Lactobacillus mindensis TMW were tested for their human probiotic potentials. Typed cultures of Staphylococcus aureus NCTC 8325 and Escherichia coli 0157:H7 were used as test pathogens. L. tucceti CECT 5920 and L. mindensis TMW had the same level of bacteriocin production and antimicrobial activity ($P \leq 0.05$). The LAB isolates resisted the pH range of 2-8 for 24 hrs while higher bile salt assimilation was shown by L. tucceti CECT 5920. Both LAB strains tolerated pepsin enzyme after 72 hrs. Cholesterol assimilation was better with L mindensis TMW. Both LAB strains did not show any haemolytic effect. L. tucceti CECT 5920 was sensitive to Cotrimoxazole while L mindensis was resistant to all the antibiotics tested. L. tucceti CECT 5920 gave better results as a LAB isolate with better probiotic potentials than L mindensis TMW.

Keywords

Bacteriocin, Fermented Food, In Vitro, LAB, Probiotic

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1. Introduction

Traditional food fermentation is a food preservation method intended to extend shelf-life, improve palatability, digestibility and the nutritive value of food by the activities of naturally occurring microorganisms especially Lactic acid bacteria (LAB) [1]. Fermentation of various foods by lactic acid bacteria (LAB) is one of the oldest forms of bio-preservation practiced by mankind. Bacterial antagonism has been recognized for over a century but in recent years this phenomenon has received more scientific attention, particularly in the use of various strains of lactic acid bacteria. One important attribute of LAB is their ability to produce anti-microbial compounds called bacteriocins [2].

An increasing interest exists for fermented food containing specific species of LAB (probiotics) with potential health-improving properties in human and animal intestinal tract [3]. Probiotics are emerging as an important new therapy for prevention and treatment of infectious diseases mainly gastrointestinal infections, deconjugation of bile salt and

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reduction in the assimilation of cholesterol in the body. This work aimed at evaluating the in vitro probiotic potentials of lactic acid bacteria isolated from traditional fermented food.

2. Materials and Methods

2.1. Sample Collection and Analyses

Twenty five samples of each traditional fermented foods: ugba, pap and kunu-zaki were purchased from the retailers at Umuahia main market while fermented cassava samples were collected from the local producers using sterile spatula into universal sterile bottles. The samples were packaged inside a cooler containing ice cubes and quickly transported to the laboratory for analyses. 1g each of the solid food samples was homogenized in 0.1% peptone water while 1 ml of kunu-zaki was serially diluted. 0.1ml aliquots of appropriate dilutions were inoculated onto De Man Rogosa and Sharpe (MRS, Oxoid, England) agar medium fortified with 50mg of nystatin [4] for the isolation of lactic acid bacteria (LAB). The plates prepared in triplicates were incubated at 35°C for 48 hrs anaerobically (using anaerobic gas packs) for isolation of mesophilic LAB. The mixed isolates were sub-cultured on MRS agar plates and the pure cultures were stored on MRS agar slants at 4°C. All the isolates were maintained by by-weekly sub-culturing on MRS agar [5].

2.2. Culture Identification

The cultures were identified by observing the colonial morphologies, microscopy, biochemical, sugar fermentation tests [5, 6] and by (GTG)5-PCR and rDNA sequencing for molecular identification.

2.3. Identification of Test Bacterial Pathogens

Typed cultures of Escherichia coli 0157:H7 and Staphylococcus aureus NCTC 8325 procured from the Veterinary Department of National Root Crops Research Institute, Umudike, Abia state were used as test isolates. They were sub-cultured onto appropriate media, gram stained and subjected to the necessary biochemical and sugar fermentation test to confirm their identities.

2.4. Screening for Bacteriocin-Producing LAB

Forty-eight LAB recovered from the fermented food samples were screened down to 10 species after gram staining, biochemical, sugar fermentation tests and molecular characterization. They were screened for bacteriocin production by the Agar Well Diffusion (AWD) assay [7] and two LAB isolates were picked as the best bacteriocin producers. These two LAB were identified as Lactobacillus tucceti CECT 5920 and Lactobacillus mindensis TMW through molecular characterization were used for further studies in this work.

2.5. Studies on Probiotic Potentials LAB

2.5.1. Determination of Antimicrobial Activity of Partially Purified Bacteriocin

The test pathogens were grown in 100ml of peptone water for 18 hr and the concentration was matched against 0.5 Mcfarland Standard to obtain a concentration of $1.0x10^6$ CFU/ml. 18 hr old culture broths of LAB isolates grown in MRS broth were centrifuged at 5000 rpm for 15 min and the pH of the cell free supernatant was adjusted to pH 6.5-7.0 with 1N NaOH to neutralize the effects of the organic acids. The LAB isolates were seeded on the surface of Mueller-Hinton Agar (Oxoid, England) using sterile swab sticks. 3mm deep wells were made on the Mueller-Hinton agar using sterile cork borer and the diluted test bacteria broths were placed into each agar well using sterile pipette. The plates were kept at room temperature for 15 minutes and then incubated at 37°C for 24 hr. The antagonistic activity of bacteriocins was determined by measuring the diameter of the inhibition zone around the wells [8].

2.5.2. Quantification of Bacteriocin Produced by LAB (Solvent Extraction)

Bacteriocin was extracted from 24 h old MRS broth of bacteriocin-producing lactic acid bacteria which was incubated at 37°C by one step solvent extraction procedure [9] with slight modification. The broth was centrifuged at 14, 000 rpm for 15 min to obtain the CFS which was then mixed with an equal volume of chloroform and agitated vigorously for 20min. It was then centrifuged at 12000 rpm for 20 min at 12°C. The precipitate formed at the interfacial region was collected by gently removing the upper solvent and then the chloroform without disturbing the interfacial components. The interfacial components in the tube were then centrifuged at 10,000 rpm for 3mins to sediment the bacteriocin. The residual chloroform was then removed by evaporation.

2.5.3. Acid Tolerance by LAB Isolates

By applying the method proposed by [10] with slight modifications, homogenized samples of lactic acid bacteria isolates were inoculated (1%, v/v) into acidified MRS broth previously adjusted to pH of 3, 4, 5, 6, 7 and 8 using 1N HCl and incubated at 35°C for 72 h. After incubation, 1 ml of the inoculated broth was serially diluted in peptone water (0.1% w/v) and pour plated [11]. The plates were incubated at 35°C for 24 h to ascertain the viability of the lactic acid bacteria. The cultures were designated positive (+) for growth and negative (-) for no growth. The control had the lactic acid

bacteria isolates incubated in MRS broth without acidification [12].

2.5.4. Bile Salt Assimilation from Culture Medium

The bacteriocin-producing LAB were evaluated for bile salt assimilation (rapidity of growth) in a broth with and without bile salts. 18 h old Lactic acid bacteria cultures were inoculated into MRS broth (1% v/v) containing 0.2, 0.3, 0.5 and 1% (w/v) concentrations of bile salt (Sodium taurocholate, Koch-Light Laboratories Ltd, England) and incubated at 35°C for 12 h. The control was prepared without bile salt [13]. After incubation, the cultures were centrifuged and unutilized bile salt in the supernatant was estimated [14]. The bile salt tolerance was determined after using the formula:

$$\%\ \text{bile salt assimilated} = \frac{(a - b)}{a} \times 100\%$$

a = initial concentration of bile salt in the medium.

b = final concentration of bile salt left in the medium after 12 hours of incubation

2.5.5. Determination of Pepsin Tolerance by LAB Isolates

The pH of 20 ml of Lactic acid bacteria's CFS was adjusted with 1N NaOH to 6.5-7.0 so as to neutralize the organic acids while the inhibitory activity of hydrogen peroxide was eliminated by the addition of 5 mg/ml catalase (c-100 bovine liver). A 5-ml aliquot of the pH adjusted CFS was aseptically transferred into test tubes and mixed with protease enzyme at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 09 and 1.0 mg/ml concentrations respectively. The test tubes with and without the enzyme (control) were incubated for 2 h at 37°C [15]. Both the control and the samples were assayed for antimicrobial activity by using well diffusion method as described earlier. The result was reported as either sensitive or resistant.

2.5.6. Cholesterol Assimilation from Culture Medium by LAB

MRS broth supplemented with 10, 20, 30, 40 and 50% concentrations (w/v) of cholesterol (BDH Laboratory Supplies, England) was inoculated with 1% of 18 h old bacteriocin-producing lactic acid bacteria broth and incubated at 35°C for 20 h [16] to determine the removal of cholesterol from culture medium. After incubation, the cultures were centrifuged and unutilized cholesterol in the supernatant was estimated [14]. The result was determined using the formula:

$$\%\ \text{cholesterol assimilated} = \frac{(a - b)}{a} \times 100$$

a = initial concentration of cholesterol in the medium.

b = final concentration of cholesterol left in the medium after 20 h of incubation.

2.5.7. Determination of Haemolytic Activity of LAB

Haemolytic activity of the LAB was evaluated on Blood agar base plates (Oxoid). Each bacterial suspension was streaked on the blood agar plates and incubated for 24 h at 37°C. After the incubation, the plates were examined for signs of β-haemolysis (clear zones around the colonies), α-haemolysis (a green-hued zone around the colonies) or γ-haemolysis (no halo around the colonies) [17].

2.5.8. Antibiotic Susceptibility of LAB

Bacteriocin-producing Lactic acid bacteria were inoculated into MRS broth (Hi-Media, India), individually and incubated for 18 h and was matched with a 0.5 McFarland standard to give a concentration of 1.0 x 10^6 CFU/ml. The pH of the broth was then adjusted to 6.5-7 using 1N NaOH to neutralize the organic acids and the inhibitory activity of hydrogen peroxide was stopped by the addition of 5 mg/ml catalase. The broth was evenly streaked with sterile swab sticks on the surface of Muller Hinton agar plates that was previously prepared and seeded with culture broth of lactic acid bacteria isolates. Antibiotic discs that contained eight antibiotics namely Cotrimoxazole (25 µg), Cloxacilin (30 µg), Erythromycin (10 µg), Gentamycin (10 µg), Augmentin (30 µg), Streptomycin (10 µg), Tetracycline (30 µg) and Chloramphenicol (30 µg) were aseptically placed upside down on the agar using sterile forceps, firmly pressed and kept for 15 min with the lid slightly opened. The plates were then incubated at 37°C for 24 h and the zones of inhibition measured [18].

2.6. Statistical Analyses

Mean separation was done using Duncan Multiple range test using Statistical Package for Social Sciences (SPSS) version 20. Differences in statistical significance were considered at $P \leq 0.05$ and n=3.

3. Results

3.1. Studies on Cultural Characteristics of LAB Isolates

3.1.1. Phenotypic Characterization of LAB Isolates from Fermented Foods

In Table 1, 48 LAB were isolated from the food samples and

were considered as LAB because they were Gram positive, catatlase negative, non-spore forming, non-motile and by sugar fermentation. These isolates were distributed into five genera namely Leuconostoc, Lactobacillus, Lactococcus, Pseudochrobactrum and Streptococcus and ten species.

Table 1. Phenotypic and biochemical identification of lactic acid bacteria isolates.

S/N	Colonial Morphology	Gram reaction	Catalase	Motility	Coagulase	Growth at 37°C	Growth at 45°C	4% NaCl	6.5% NaCl
1	Flat, creamy Mucoid	+rods	-	-	-	+	-	+	-
2	Circular, milky, Mucoid	+rods	-	-	-	+	+	+	-
3	Creamy, Mucoid	+cocci	-	-	-	+	-	-	-
4	Circular, milky, slimy	+cocci	-	-	-	+	-	-	+
5	Circular, creamy, convex	+rod	-	-	-	+	+	+	+
6	Circular, milky, slimy	+cocci	-	-	-	+	+	-	-
7	Circular, milky, Mucoid	+rod	-	-	-	+	+	-	-
8	Flat, milky, slimy	+rod	-	-	-	+	+	+	+
9	Circular, creamy, convex	+rod	-	-	-	+	+	-	-
10	Flat, membranous	+rod	-	-	-	+	+	-	-

S/N	Colonial Morphology	Gram reaction	Glucose	Sucrose	S/N	Maltose	Fructose	Lactose	Mannitol
1	Flat, creamy Mucoid	+rods	+	+	1	+	+	+	+
2	Circular, milky, Mucoid	+rods	+	+	2	+	+	+	+
3	Creamy, Mucoid	+cocci	+	+	3	+	+	+	+
4	Circular, milky, slimy	+cocci	+	+	4	+	+	+	+
5	Circular, creamy, convex	+rod	+	+	5	+	+	+	+
6	Circular, milky, slimy	+cocci	+	+	6	+	-	+	+
7	Circular, milky, Mucoid	+rod	+	-	7	+	+	+	+
8	Flat, milky, slimy	+rod	+	-	8	+	-	+	+
9	Circular, creamy, convex	+rod	+	+	9	+	+	+	+
10	Flat, membranous	+rod	+	+	10	-	+	+	+

S/N	Colonial Morphology	Gram reaction	Galactose	Saccharose	Oxidase	Methyl red	Gas production	V. P	Isolate
1	Flat, creamy Mucoid	+rods	+	-	-	+	-	-	Lactobacillus casei
2	Circular, milky, Mucoid	+rods	+	-	-	+	-	-	Pseudochrobactrum asaccharolyticum
3	Creamy, Mucoid	+cocci	+	-	-	+	-	-	Pseudochrobactrum saccharolyticum
4	Circular, milky, slimy	+cocci	+	-	-	+	-	-	Leuconostoc mesenteroides
5	Circular, creamy, convex	+rod	+	-	-	+	-	-	Lactococcus plantarum
6	Circular, milky, slimy	+cocci	+	-	-	+	-	-	Streptococcus thermophilus
7	Circular, milky, Mucoid	+rod	+	-	-	+	-	-	Lactobacillus acidophilus
8	Flat, milky, slimy	+rod	+	-	-	+	-	-	Lactobacillus helveticus
9	Circular, creamy, convex	+rod	+	-	-	+	-	-	Lactobacillus tucceti CECT 5920
10	Flat, membranous	+rod	+	-	-	+	-	-	Lactobacillus mindensis TMW

Positive reaction (+), Negative reaction (-)

3.1.2. Molecular Identification of LAB Isolates

Figure 1 shows the Gel electrophoretic result of the four presumptive bacteriocin-producing LAB identified as Pseudochrobactrum asaccharolyticum, P. saccharolyticum, L. tucceti cect 5920 and L. mindensis TMW

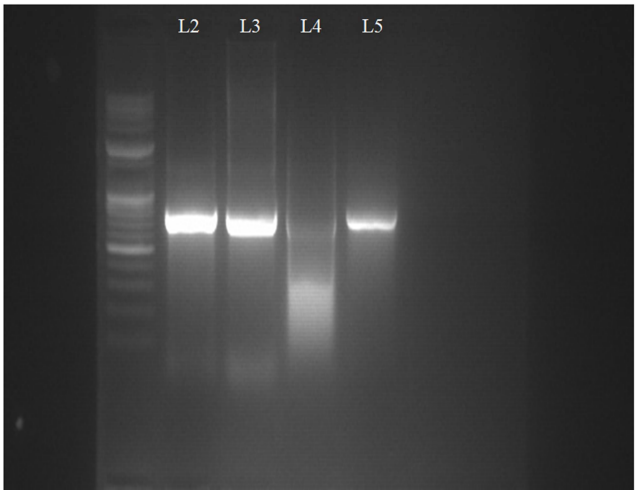

Figure 1. Electrophoretic bands of the four bacteriocin-producing LAB.

L2: Pseudochrobactrum asaccharolyticum

GCGTCATAACAGAACAGACACCCGCC

TTCGCCACTGGTGATCCTGCTATCTACGAATTTCACCGCTACACAG

GAATTCTACTTACCTCTATATTACTCAAGCTCTGCAGTATCCAAGGCACT

TTCCCGGTTGAGCTAGGAATTTCACTCTGACTTAAAAAACCGCCTACG

AACGCTTTACACCCAATAAATCCGGACAACGCTCGCATCCTACGTATTAC

CGCGGCTGCTGGCACGGAGTTAGCCGAGGCTTTTTCGTAGAGTACCGTCA

AGACCCTAACCGTAGGGAGGATTCTTCTTGTACAAAAACAACTTAAATTC

CATAGCACGAACCCCTTGCGCGCGGCACGGCTGGGCCACAGTCGCCTCTG

TTGCCTAGTATAAGATTCTGCAGCGTCGCCTACGAGTCGGGTGCGGGTCT

CGTCACCAGCTGGGGGATCTAACTCCCCTGACCCGTAAGCATCGTTGCC

TTGGTATGGCGAGACCACCCCCGCTAATGATAAACATGCCGTC

ATACCGAGAAATGATTACATATATGCCATATCGATAAACCATGG

AGCATTAATACGAATTTCTTCAGGCTATTCCCCTGTATAAGGCAAGTTGC

AGACCCGTTACTCACCCGTGCGCCGGTCTCCAACAGCATGCTCATG

L3: Pseudochrobactrum saccharolyticum

TTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTAATGGACCAGTAA

GCCGCCTTCGCCACTGGTGTTCCTGCGAATATCTACGAATTTCACCTCTA

CACTCGCAATTCCACTTACCTCTTCCATACTCAAGACTTCCAGTATCAAA

GGCAGTTCCGGGGTTGAGCCCCGGGATTTCACCCCTGACTTAAAAGTCCG

CCTACGTGCGCTTTACGCCCAGTAAATCCGAACAACGCTAGCCCCCTTCG

TATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGTTAC

CGTCATTATCTTCACCGGTGAAAGAGCTTTACAACCCTAGGGCCTTCATCA

CTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCA

CTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGSTGA

TCATCCTCTCAGACCAGSTATGGATCGTCGCCTTGGTAGGCCTTTACCCTA

CCAACTAGGTAATCCAACATGGGCTCATCATTCTCCGATAAATCTTTCCCC

AAAAGGGCGTATACGGTATTAGCACAA

L4: Lactobacillus tucceti cect 5920

GCGTCAGTTACAGACCAGAAAGCCGCCTTCGCCACTGGTGTTCT

TCCATATATCTACGCATTTCACCGCTACACATGGAGTTCCACTT

TCCTCTTCTGCACTCAAGTTTACCAGTTTCCGAAGCACTTCCTC

GGTTGAGCCGAGGGCTTTCACTTCAGACTTAAAAAACCGCCTAC

GTTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCCCTACG

TATTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTTCTGGT

TGAATACCGTCAATACGTGAACAGTTACTCTCACACATGTTCTT

CTTCAACAACAGAGTTTTACGAGCCGAAAACCTTCTTCACTCAC

GCGGCTGTGCTCCATCAGGCTTTCGTCCATTGTGGAAGATTCCG

TACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAA

TGTGGCCGATTACCCTCTCAGGTCGGCTACGTATCATTGCCTTG

GTGAGCCGTTACCTCACCAACTAGCTAATACNCCGCGGGTCCAT

CCNAAAGCGATAGCAGAACCATCT.

L5: Lactobacillus mindensis TMW

ACTACAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCA

GCGTCAGTAATGGACCAGTAAGCCGCCTTCGCCACTGGTGTTCCTGC

GAATATCTACGAATTTCACCTCTACACTCGCAATTCCACTTACCTCT

TCCATACTCAAGACTTCCAGTATCAAAGGCAGTTCCGGGGTTGAGCC

CCGGGATTTCACCCCTGACTTAAAAGTCCGCCTACGTGCGCTTTACG

CCCAGTAAATCCGAACAACGCTAGCCCCCTTCGTATTACCGCGGCTG

CTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGTTACCGTCATTATC

TTCACCGGTGAAAGAGCTTTACAACCCTAGGGCCTTCATCACTCACG

CGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCCCT

3.1.3. Diversities of LAB Isolates

In Table 2, the diversity of the ten LAB isolates is presented with the highest number of LAB isolates (7) from ugba while the lowest number of isolates (3) was from fermenting cassava. L. mindensis TMW was the most isolated LAB (38%) while L. plantarum was the least (6.4%) from the four food samples.

Table 2. Microbial diversity in fermented foods and bacteriocin activity.

S/No	Fermented food	Lactic acid bacteria isolate	Number of species/percentage	Bacteriocin activity
1	Cassava	Pseudochrobactrum saccharolyticum	10(4.0)	+
		Lactobacillus acidophilus	5(2.0)	-
		Lactobacillus helveticus	8(3.2)	-
		Lactobacillus casei	18(7.2)	-
2	Ugba	Pseudochrobactrum asaccharolyticum	17(6.8)	+
		Leuconostoc mesenteroides	16(6.4)	-
		Lactococcus plantarum	8(3.2)	-
		Lactobacillus acidophilus	10(4)	-
		Lactobacillus tucceti CECT 5920	12(4.8)	+++
		Lactobacillus mindensis TMW	20(8.0)	+++
3	Ogi	Pseudochrobactrum asaccharolyticum	17(6.8)	+
		Lactococcus plantarum	8(3.2)	-
		Streptococcus thermophilus	9(3.6)	-
		Lactobacillus acidophilus	10(4.0)	-
		Lactobacillus tucceti CECT 5920	10(4.0)	+++
		Lactobacillus mindensis TMW	18(7.2)	+++
4	Kunu-zaki	Pseudochrobactrum saccharolyticum	10(4.0)	+
		Leuconostoc mesenteroides	16(6.4)	-
		Streptococcus thermophilus	9(3.6)	-
		Lactobacillus helveticus	20(8.0)	-

Key: (-) No inhibition; (+) Low inhibition; (+++) High inhibition.

3.2. Studies on Probiotic Potentials LAB Isolates

3.2.1. Quantification of Bacteriocin Produced by LAB Isolates

Table 3 shows the bacteriocin production by the two LAB. L. tucceti CECT 5920 produced 0.06 mg/ml of bacteriocin while L. mindensis TMW produced 0.08 mg/ml of bacteriocin.

Table 3. Quantification of bacteriocin produced by lactic acid bacteria isolates.

Sample	Quantity (mg/ml)	Isolate
1	0.06±0.02	Lactobacillus tucceti CECT 5920
2	0.08±0.02	Lactobacillus mindensis TMW

Values are means of three replicates ± standard deviation (SD).

3.2.2. Antimicrobial Activity of Partially Purified Bacteriocin Extract

Table 4 shows that the antimicrobial activity of partially purified bacteriocin extract of L. tucceti CECT 5920 statistically had same level of inhibitory activity against both test bacteria. L. mindensis TMW also had equal level of antimicrobial activity against the two test pathogens.

Table 4. Antimicrobial activity of partially purified bacteriocin (mm).

Lactobacillus tucceti CECT 5920		Lactobacillus mindensis TMW	
S. aureus	E. coli	S. aureus	E. coli
19±0.08[a]	18±0.08[a]	18±0.08[a]	17±0.07[a]
*Control	0	0	0

[a]Data values with the same letters are not significantly different (P≤0.05; n=3).
*Control: MRS broths without LAB isolates

INTERPRETATIVE REFRENCE RANGE

SENSITIVE INTERMEDIATE RESISTANT

≥1711 – 15 ≤10

3.2.3. Acid Tolerance of LAB Isolates

In Table 5, result shows that the two LAB tolerated the pH of the medium of 3-8 for 10 h of incubation.

Table 5. Acid tolerance of LAB isolates.

	L. tucceti CECT 5920 Time (h)					L. mindensis TMWTime (h)				
pH	**2**	**4**	**6**	**8**	**10**	**2**	**4**	**6**	**8**	**10**
3.0	+	+	+	+	+	+	+	+	+	+
4.0	+	+	+	+	+	+	+	+	+	+
5.0	+	+	+	+	+	+	+	+	+	+
6.0	+	+	+	+	+	+	+	+	+	+
7.0	+	+	+	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+	+	+	+

Key: + means growth.

3.2.4. Determination of Bile Salt Resistance by LAB Isolates

Figure 2 shows the bile salt resistance of the two LAB in broths containing 0.2-1.0% ox-bile salt. L. tucceti CECT 5920 showed 54.06% resistance at 0.30% concentration while L. mindensis TMW showed less than 54.06% resistance at 0.30% concentration.

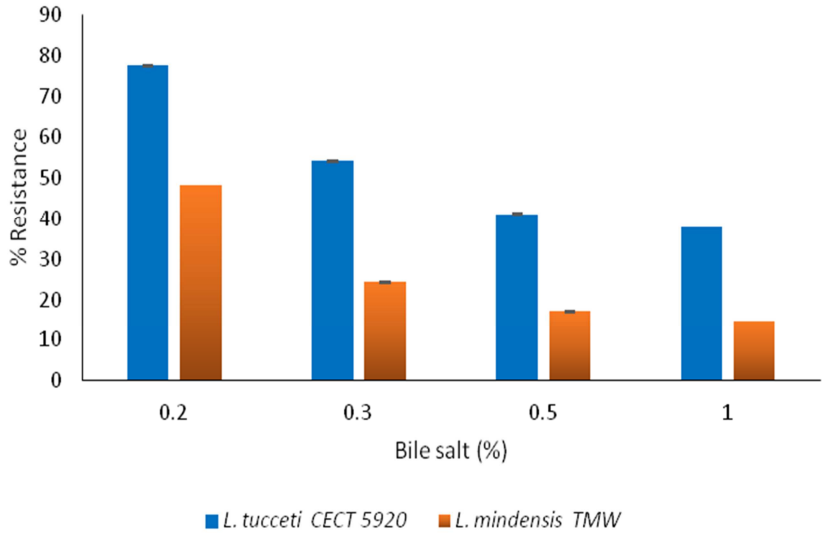

Figure 2. Determination of bile salt resistance by LAB isolates (%).

3.2.5. Pepsin Tolerance by LAB Isolates

Table 6 shows that when the two LAB were cultured in the presence of pepsin enzyme at 0.1-1.0 mg/ml concentrations, they showed moderate growth after 24 hrs and heavy growth after 72 hrs. Pepsin enzyme did not stop the growth of the LAB isolates.

Table 6. Pepsin tolerance by LAB isolates.

ISOLATES	**VIABILITY OF ISOLATES**					
	WITH ENZYME			**CONTROL (NO ENZYME)**		
	24 hr	**48 hr**	**72 hr**	**24 hr**	**48 hr**	**72 hr**
L. tucceti (strain CECT 5920)	++	+++	+++	+++	+++	+++
L. mindensis (strain TMW)	++	++	+++	+++	+++	+++

KEY: + Scanty growth; ++ Moderate growth, +++ Heavy growth

3.2.6. Cholesterol Assimilation from Culture Broths by LAB Isolates

In Figure 3, L. mindensis TMW absorbed 15.91 mg/ml from the growth medium while L. tucceti CECT 5920 absorbed 6.21 mg/ml both at 30% concentration.

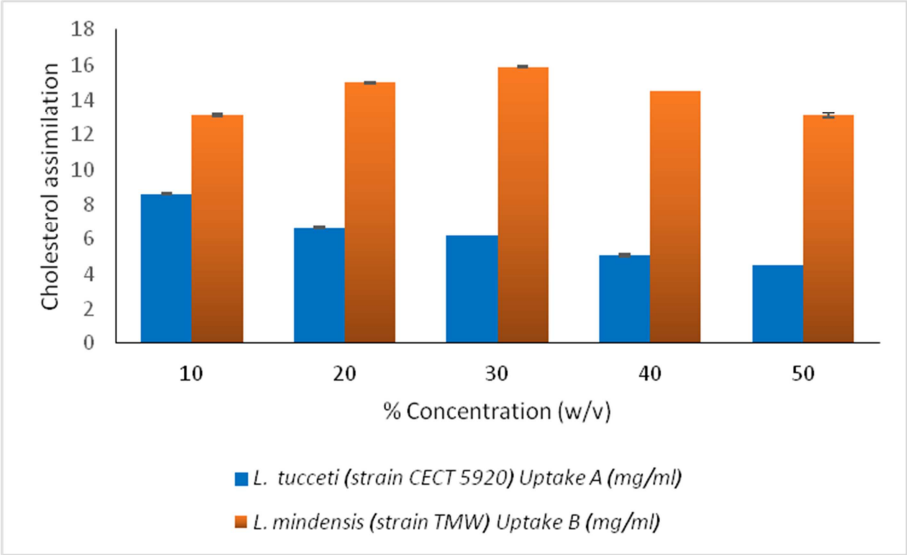

Figure 3. Cholesterol uptake by LAB isolates (mg/ml).

3.2.7. Haemolytic Activity of LAB Isolates

Table 7 shows that none of the two LAB showed any haemolytic activity on blood agar.

Table 7. Haemolytic effect of LAB isolates.

Isolate	Reaction (Haemolysis)
L. tucceti (strain CECT 5920)	γ-haemolysis (no halo around colonies)
L. mindensis (strain TMW)	γ-haemolysis (no halo around colonies)

3.2.8. Antibiotic Susceptibility Pattern of LAB Isolates

Table 8 shows that L. tucceti CECT 5920 was sensitive to cotrimoxazole out of the eight antibiotics while L. mindensis TMW was resistant to the eight antibiotics.

Table 8. Antibiotic susceptibility pattern of LAB isolates (mm).

	Cotrimoxazole (25µg)	Cloxacilin (30µg)	Erythromycin (10µg)	Gentamycin (10µg)	Augmentin (30µg)	Streptomycin (10µg)	Tetracycline (30µg)	Chloramphenicol (30µg)
L. tucceti CECT 5920	18.06±0.08	12.02±0.02	8.10±0.14	8.02±0.02	8.06±0.08	8.07±0.09	8.02±0.03	10.06±0.08
L. mindensis TMW	8.05±0.07	8.06±0.09	9.06±0.09	9.02±0.03	8.07±0.09	8.06±0.08	8.01±0.01	8.02±0.02

Values are means of three replicates ± standard deviation (SD).

INTERPRETATIVE REFERENCE RANGE		
SENSITIVE	**INTERMEDIATE**	**RESISTANT**
≥15	13 – 14	≤12

4. Discussion

The present investigation was aimed at determining the in vitro probiotic potentials of lactic acid bacteria isolated from traditional fermented food samples. Results showed that 48 isolates were recovered from the 100 samples of traditional fermented food collected. The preliminary identification of isolated lactic acid bacteria was in agreement with almost each and every study on lactic acid bacteria preliminary identification [19]. Non-production of gas by the isolates suggests that they are homo-fermentative lactic acid bacteria producing only organic acids without gas in growth medium.

Lactobacillus, Leuconostoc, Lactococcus and Streptococcus have been isolated from traditional fermented foods in the past [20, 21] by morphological and biochemical characterization. However, Lactobacillus tucceti CECT 5920, L. mindensis TMW, Pseudochrobactrum asaccharolyticum and P. saccharolyticum have not been reported before now as lactic acid bacteria isolated either from Nigerian traditional fermented foods or fermented foods from any other part of the world. This could be due to the type of fermented foods used or the environment of the study.

The highest in diversity of isolates shown by L. mindensis

TMW in all the traditional fermented food samples indicates that this isolate has become ecologically and physiologically adapted to the fermentation of these food samples. Ugba having the highest diversity of lactic acid bacteria could be due to its high proteinous content which supported the greatest number of lactic acid bacteria compared with the other traditional fermented foods sampled. However, contrary to this finding, [22] showed that Pediococcus acidilactici, L. plantarum, L. fermentum, L. lactis and Leuconostoc mesenteroides as the most divers lactic acid bacteria isolated from traditionally fermented maize gruels from five different western states of Nigeria respectively

Some factors might have influenced the low number of isolation of bacteriocin-producing lactic acid bacteria such as the culture medium, incubation conditions and genetic make-up of the isolate, or the sensitivity methods used in determining the antimicrobial activity of the isolates. It was reported that cell aggregation and medium composition can affect bacteriocin production by LAB [23].

The presence of one band on each of the four ladders indicates that the DNA strands of the four isolates were cut once with suitable restriction endonucleases. The isolation of L. pentosus having 260 bp using 16S rRNA analysis has been reported [24] and this result is close to L. tucceti CECT 5920 isolate that has 250 bp. Their results also showed that L. plantarum with subspecies unidentified, L. plantarum subsp. argentoratensis. L. plantarum with subspecies unidentified and Lactobacillus plantarum subsp. plantarum had about the same molecular weight of 550 bp and this result is close to P. asaccharolyticum, P. saccharolyticum and L. mindensis TMW that have molecular weight of about 520 bp.

Assessment of bacteriocin production by the two isolates showed that they have statistically similar results. Bacteriocin production is considered an important probiotic attribute of lactic acid bacteria in the present search for human probiotic LAB.

Bacteriocins produced by these isolates have broad spectrum activity against gram positive and gram negative bacteria (S. aureus and E. coli respectively). Similar results were recorded by [25] against S. aureus, E. coli and Pseudomonas aeruginosa. Their antagonistic property is attributed to the low pH, the un-dissociated acid and production of other primary and secondary antimicrobial metabolites [26]. Lactic acid bacteria have potentials to inhibit the growth of pathogenic and spoilage bacteria and the possibilities exist for their use as probiotic LAB.

The tolerance of the isolates to low pH proves they can be used as human probiotics as they can tolerate the acidic nature of the stomach during movement to the intestine. It was reported that the strains of lactic acid bacteria tested by [27] tolerated three hours of acid exposure to pH 2 and 3 and this is in agreement with the findings from this research. This finding is also in agreement with the reports of [28, 29 and 30]. Low acidity is known as the most negative factor that effects the growth and viability of lactobacilli during their passage through the stomach [28]. Because, the pH in human stomach ranges from 1.5 to 4.5 depending on the intervals of feeding or the food variability, and from the duration of food digestion which can take up to 3 hr, some authors proposed that strains intended for probiotic purposes should be screened according to their tolerance to pH 2.5 in an HCl-acidified culture medium during four hours [31, 32].

The bile salt lowering effect recorded by the LAB isolates in this work is an indication of their probiotic potential and a good biotechnological trait. According to [33], the concentration 0.3% bile salts is considered as critical for resistant strains screening and the same level is critical for the human probiotics selection. Because of their similarity, a value of 0.3% Oxgall (Ox-bile) solution is the most used to substitute human bile salts [34]. Bile salt assimilation is an important probiotic potential of LAB in view of literature support that has accredited bile salt assimilation to some human probiotic bacteria. The bile salt-lowering effect of Lactobacillus spp. is by several means through bile salt hydrolase (BSH) activity [35]. BSH is the enzyme responsible for bile salt de-conjugation during entero-hepatic circulation [36]. Bile is a result from a digestive secretion that can play a capital role in the lipids emulsification and has the ability to affect the phospholipids, cell membranes proteins and disrupt cellular homeostasis [30]. The probiotic and bile salt-lowering property of Lactobacillus spp. isolated from non human origin was evaluated by [37]. Four strains tolerated 0.30% (w/v) bile salt during the 2 hrs of growth and this result is in agreement with the findings here. [27] found that ten strains of lactic acid bacteria out of the lot they worked on showed resistance to 0.3% of ox-bile (percentage of resistance ≥ 50%) and this report also is in agreement with the performance of L. tucceti CECT 5920 from this work.

The tolerance showed by the LAB isolates to pepsin enzyme also indicates that the two isolates possess another important potential required of suitable probiotic LAB for use in human body.

The Lab isolates were able to take up cholesterol from the medium. [37] reported a reduction in cholesterol levels from resting and dead cell free broth of all 4 strains that ranged between 13.11-23.28 and 11.44 -19.53%, respectively and this report agrees with the findings of this work from L.

mindensis TMW. The ability of the organism to reduce the cholesterol level was due to assimilation of cholesterol within bacterial cell [38] and increased excretion of bile salts due to de-conjugation by the bile salt hydrolase [39] into amino acid residues and free bile salts. Elevated level of certain blood lipids are a greater risk for cardiovascular disease. A few research reports describe the use of L. acidophillus to decrease the serum cholesterol levels in human and animals. The hypocholesteromic potential exhibited by the LAB isolates used in this work is an indication that they will perform well in the area of cholesterol reduction when used as human probiotic.

The two LAB did not exhibit haemolytic activity. This result is in agreement with [40] who had reported that none of the Lactobacillus plantarum strains they worked on produced haemolysin on sheep blood. [27] did not observe any strain of LAB that exhibited β-haemolytic activity (clear zones around colonies) when grown in Columbia blood agar. They also found that while most strains were γ-haemolytic (no halo around colonies), only two strains LPAR3 and LPAR12 exhibited α-haemolytic activity (a green-hued zone around colonies) [17]. The non-haemolytic attributes of the two LAB used in this work suggests that the isolates could be good candidates as human probiotic isolates.

One of the main criteria needed to be fulfilled by a probiotic organism is that it should be non-pathogenic [41, 42]. Result from this work showed that the LAB isolates will resist broad spectrum antibiotic therapy when they are used as probiotics in the intestine thus ensuring their survival and establishment as part of the microflora so as to elicit their antagonistic effects on the intestinal pathogens. Among antibiotic resistances, vancomycin resistance is of major concern because vancomycin is one of the last antibiotics broadly efficacious against clinical infections caused by multidrug resistant pathogens. Some LAB however, including strains of L. casei, L. plantarum and Leuconostoc spp., L. bulgaricus, L. fermentum were found to be resistant to vancomycin. Such resistance is usually intrinsic, that is, chromosomally encoded and non-transmissible [43]. L. mindensis TMW was completely resistant to the eight antibiotics tested (Table 8). [44] reported that almost all the strains of lactic acid bacteria they tested were resistant to penicillin and 10% were susceptible to ampicillin, (β-lactam antibiotics). [44] had earlier reported that Lactobacillus and Bifidobacterium strains were susceptible to β-lactam antibiotics (penicillin, ampicillin) and this may be due to the difference in source of isolation.

5. Conclusion

The study on the in vitro screening for human probiotic potential of the two lactic acid bacteria: L. tucceti CECT 5920 and L. mindensis TMW revealed that both isolates had rapid acidification ability as well as production of bacteriocin with varied levels of antimicrobial activities against the two test pathogens: S. aureus NCTC 8325 and E. coli 0157:H7 used in this work. Much of the interest in the analysis of LAB produced bacteriocins is driven by their potential applications. The LAB grew well over a wide pH range, were not inhibited by pepsin enzyme, assimilated bile salt and cholesterol from growth medium and did not lyse red blood cell. L. tucceti CECT 5920 did better than L. mindensis TMW, however, both LAB isolates would make good research organisms of study for in vivo studies as human probiotic LAB.

6. Recommendation

The isolation of LAB with probiotic potentials from traditional fermented foods indicates that these foods possess human probiotic values. Hence, their consumption is encouraged beyond the traditional localities.

References

[1] Zeuthen P (1995). Historical aspects of meat fermentations. In: G. Campbell-Platt and P. E. Cook (ed.), Fermented meats. Blackie Academic and Professional, London, United Kingdom. Pp 53-68.

[2] Lindgren, S. E. and Dobrogosz, W. J. (1990). Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiology Review, 7(1-2):149-63.

[3] Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U. and Huis in't veld, J. H. J. (1998). Overview of gut flora and probiotics. International Journal of Food Microbiology 41, 85–101.

[4] Cheesbrough M (2004) District Laboratory Practice in Tropical Countries. Part 2, Cambridge University Press UK. Pp: 253-269.

[5] Roissart, H. and Luguet, F. M. (1994). Bacteries lactiques. Aspects fondamentaux et technologiques. Uriage, Lorica, by Lactococcus lactis subsp cremoris B40. Carbohydrate Polymers 37: 123-130.

[6] Bergey, D. H. (1923) John G. Holt, Peter H. Sneath (eds) Bergey's Manual of Systematic Bacteriology (Vol. 2).

[7] Lasta, S., Fajloun, Z., Darbon, H., Mansuelle, P., Andreotti, N., Sabatier, J., Abdellatif, L., Boudabous., A. and Sampieri, F. (2008). Chemical synthesis and characterization of J46 peptide, an atypical class IIa bacteriocin from Lactococcus lactis subsp. Cremoris J46 strain. Journal of Antibiotics 61: 89-93.

[8] Joshi, V. K., Somesh, S. and Neerja, S. R. (2006). Production, Purification, Stability and Efficacy of Bacteriocin from Isolates of Natural Lactic Acid Fermentation of Vegetables. Food Technology Biotechnology 44 (3) 435-439.

[9] Burianek, L. L and Youssef, A. E. (2000). Solvent extraction of bacteriocins from liquid cultures. Letters in Applied Microbiology, 31: 193-197.

[10] Pereira, D. I., Dora, I. A. and Gibson, G. R. (2002). Cholesterol assimilation by lactic acid bacteria and bifidobacteria isolated from the human gut. Applied Environmental Microbiology 68: 4689- 4693.

[11] Dave, R. I and Shah, N. P. (1996). Evaluation of media for selective enumeration of Streptococcus thermophilus, Lactobacillus delbrueckii ssp. bulgaricus, Lactobacillus acidophilus and bifidobacteria. Journal of Dairy Science, 79, 1529–1536.

[12] Md, H., Kaname, T., Minoru, U. and Taku, M. (2007). Probiotic characteristics of Lactic Acid Bacteria Isolated from Traditional Fermented Milk 'Dahi' in Bangladesh. Pakistan Journal of Nutrition 6 (6): 647-652.

[13] Dora, I. A., Pereira, P. and Glenn, R. G. (2002). Cholesterol Assimilation by Lactic Acid Bacteria and Bifidobacteria Isolated from the Human Gut. Applied and Environmental Microbiology. 8: 4689–4693.

[14] Zlatkis, A., Zak, B. and Boyle, A. J. (1953). A new method for the direct determination of serum Cholesterol, Journal of Laboratory Clinical Medicine 41: 486-492.

[15] Rajaram, G., Manivasagan, P., Thilagavathi, B. and Saravanakumar, A. (2010). Purification and Characterization of a Bacteriocin Produced by Lactobacillus lactis Isolated from Marine Environment. Advance Journal of Food Science and Technology 2(2): 138-144.

[16] Liong, M. T. and Shah, N. P. (2005). Acid and bile tolerance and cholesterol removability of lactobacilli strains, Journal of Dairy Science 88:55-66.

[17] Maragkoudakis, P. A., Konstantinos, C. M., Psyrras, D., Cremonese, S., Fischer, J., Cantor, M. D. and Tsakalidou, E. (2009). Functional properties of novel protective lactic acid bacteria and application in raw chicken meat against Listeria monocytogenes and Salmonella enteriditis. International Journal of Food Microbiology 130: 219–226.

[18] Vijai, P., Marilingappa, J. and Kadirvelu, J. (2004). Isolation and characterization of Bacteriocin producing lactic acid bacteria from A south indian special dosa (appam) batter Journal of Culture Collections 4: 53-60.

[19] Sudi, I. Y., De, N. and Ali-Dunkara, U. (2008). Mutagenesis and Selection of Lactobacillus bulgaricus and Streptococcus thermophilus for ppotential use as starter culture. Journal of American Sciences, 4(3): 80-87.

[20] Oyedeji, O., Ogunbanwo, S. T. and Onilude, A. A. (2013). Predominant Lactic Acid Bacteria Involved in the Traditional Fermentation of Fufu and Ogi, Two Nigerian Fermented Food Products. Food and Nutrition Sciences, 4: 40-46.

[21] Oyewole, O. A. and Isah, P. (2012). Locally Fermented Foods in Nigeria and their Significance to National Economy: a Review. Journal of Recent Advances in Agriculture, 1(4): 92-102.

[22] Muller RA (2000). Characterization of the microbial ecosystem of cereal fermentations using molecular biological methods, pp. 1-24.

[23] Wang, Q., Cui, Y., Lackeyram, D., Yuan, L., Xu, J., Wang, W. and Li Xu, L. (2010). Effect f cultural components on antimicrobial activity of bacteriocin produced by bacteria isolated from gut of poultry. African Journal of Microbiology Research, 4(19): 1970-1980.

[24] Agaliya and Jeevaratnam (2013) (2013) Molecular characterization of lactobacilli isolated from fermented idli batter. Brazilian Journal of Microbiology 44, 4, 1199-1206.

[25] Adesokan, I. A., Odetoyinbo, B. B and Okanlawon, B. M. (2009). Optimization of Lactic Acid Production by Lactic Acid Bacteria Isolated from Some Traditional Fermented Food in Nigeria. Pakistan Journal of Nutrition 8 (5): 611-615.

[26] Ten Brink, B., Minekus, M., Vander Vossen, J. M., Leer, R. J. and Huis, J. H. (1994). Antimicrobial activity of lactobacilli: Preliminary characterization and optimization of production of acidocin B. a novel bacteriocin produced by Lactobacillus acidophilus M46. Journal of Applied Bacteriology 77: 140-148.

[27] Naoual, J., Abdelaziz, B. and Mohammed, B. (2011). Probiotic Potential of Lactobacillus strains Isolated from Known Popular Traditional Moroccan Dairy Products. British Microbiology Research Journal. 1(4): 79-94.

[28] Charteris, W. P., Kelly, P. M., Morelli, L. and Collins, J. K. (1998). Development and appllication of an in vivo methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. Journal of Applied Microbiology, 84, 759–768.

[29] Petros, A., Maragkoudakis, P. A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B. and Tsakalidou, E. (2006). Probiotic potential of Lactobacillus strains isolated from dairy products. International Dairy Journal, 16, 189-199.

[30] Burns, P., Patrignani, F., Serrazanetti, D., Vinderola, G. C., Reinheimer, J. A., Lanciotti, R and Guerzoni, M. E. (2008). Probiotic Crescenza cheese containing Lactobacillus casei and Lactobacillus acidophilus manufactured with high-pressure homogenized milk. Journal of Dairy Sciences, 91: 500-512.

[31] Pennacchia, C., Ercolini, D., Blaiotta, G., Pepe, O., Mauriello, G. and Villani, F. (2004). Selection of Lactobacillus strains from fermented sausages for their potential use as probiotics. Meat Science, 67: 309-317.

[32] Klingberg, T. D., Axelsson, L., Naterstad, K., Elsser, D. and Budde, B. B. (2006). Identification of potential probiotic starter cultures for Scandinavian- type fermented sausages. International Joournal of Food Microbiology, 105: 419–431.

[33] Gilliland, S. E., Staley, T. E and Bush, L. J (1984). Importance of bile tolerance of Lactobacillus acidophilus used as a dietary adjunct. J Dairy Sci; 67(12):3045-51.

[34] Brashears, M. M., Galyean, M. L., Loneragan, G. H and Killinger-Mann, K. (2003). Prevalence of Escherichia coli O157, H7 and performance by beef feedlot cattle given Lactobacillus direct-fed microbials. Journal of Food Protection, 66, 748-754.

[35] Begley, M., Hill, C and Gahan, C. G. M (2006) Bile Salt Hydrolase Activity in Probiotics Applied and Environmental Microbiology 72(3):1729-1738.

[36] Moser, S. A. and Savage, D. C. (2001). Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in lactobacilli. Applied Environmental Microbiology, 67: 3476-3480.

[37] Sasithorn, S., Chaiyavat, C., Duangporn, K. and Plearnpis, L. (2010). Characterisation of non human origin probiotic Lactobacillus plantarum with cholesterol-lowering Property. African Journal of Microbiology Research. 4 (10): 994-1000.

[38] Lavanya, B., Sowmiya, S., Balaji, S. and Muthuvelan, B. (2010). Screening and Characterization of Lactic Acid Bacteria from Fermented Milk. British Journal of Dairy Sciences 2(1): 5-10.

[39] Salminen, M. K., Tynkkynen, S., Rautelin, H., Saxelin, M., Vaara, M., Ruutu, P., Sarna, V., Valtonen, A. and Järvinen, D. (2002). Lactobacillus bacteremia during a rapid increase in probiotic use of Lactobacillus rhamnosus GG in Finland. Clinical Infectious Diseases, 35, 1155-1160.

[40] Kacem, M. and Karam, N. E. (2006). Physicochemical and microbiological study of "shmen", a traditional butter made from camel milk in the sahara (Algeria): isolation and identification of lactic acid bacteria and yeasts. Pure Sciences 57: 198-204.

[41] Dubois, M. K., Gilles, A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956). Colorimetric Method for determination of sugars and related substances. Analytical Chemistry, 28(3): 350-356.

[42] Ljungh, A. and Wadstrom, T. (2006). Lactic acid bacteria as probiotics. Current Issues in Intestinal Microbiology, 7: 73-89.

[43] Zhou, J. S., Shu, Q., Rutherfurd, K. J., Prasad, J., Birtles, M., Gopal, P. K. and Gill, H. S. (2000b). Safety assessment of potential probiotic lactic acid bacterial strains Lactobacillus rhamnosus HN001, Lb. acidophilus HN017, and Bifidobacterium lactis HN019 in BALB/c mice. International Journal of Food Microbiology, 56: 87-96.

[44] Zhou, J. S., Gopal, P. K. and Gill, H. S. (2000a). Potential probiotic lactic acid bacteria Lactobacillus rhamnosus (HN001), Lactobacillus acidophilus (HN017) and Bifidobacterium lactis (HN019) do not degrade gastric mucin in vitro. International Journal of Food Microbiology, 63: 81-89.